Isolation of predominantly submicron-sized UHMWPE wear particles from periprosthetic tissues

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A method of tissue digestion using sodium hydroxide was applied to the isolation and recovery of ultra-high-molecular-weight polyethylene (UHMWPE) particles from tissues around failed total hip replacements. Density gradient ultracentrifugation of the digested tissues was performed to separate the UHMWPE from cell debris and other particulates. Fourier transform infrared spectroscopy and differential scanning calorimetry (DSC) verified that the recovered particles were UHMWPE. When viewed by scanning electron microscopy, individual particles were clearly observed and were either rounded or elongated. The majority were submicron in size. The application of this method to the study of particles from periprosthetic tissues may elucidate aspects of biomaterial particle size and shape that are important to the biologic response to, and clinical outcome of, total joint replacement. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Wear particles produced at the articulating surfaces of total joint prostheses have been implicated as the cause of inflammatory bone loss and implant loosening.[1-6] The relative roles of particle shape, size, material, and number in this process remain unclear, in part because of the difficulty in characterizing intracellular particulates. Histologic examination of wear particles is limited for the following reasons. First, light microscopy is limited to particles that are large enough to be resolved by the optics of the microscope used, generally >1 μm. Second, tissue processing and sectioning can affect the final appearance of the particles. For example, bone cement is dissolved by the clearing agents used in paraffin processing, and the angle at which a particle is sectioned may result in the misinterpretation of its true shape. Last, particles must be recognized within the sections, so observer bias or experience may be a factor in the assessment of debris in tissues.

An alternative is the isolation of the particles from the tissues and their subsequent examination by scanning electron microscopy (SEM) or automated measuring techniques. Several methods of tissue digestion have been reported for the recovery of metallic or bone cement particles.[7,8] The high density of those particles facilitates their recovery from tissues because centrifugation will cause them to pellet out at the bottom of the solution. The recovery of polyethylene particles, which have a density less than that of water (0.93–0.94 g/cm^3), required new methods to be devised through a series of experiments with various reagents and separation techniques.[9] Because uniformly sized and shaped ultra-high-molecular-weight polyethylene (UHMWPE) particles were not available, those experiments were performed by recovering high-density polyethylene (HDPE) beads (Shamrock, NJ) that had been added to tissues harvested from patients undergoing primary joint replacement surgery. A method based on sodium hydroxide tissue digestion and ultracentrifugation over a sucrose gradient successfully recovered the HDPE particles for SEM examination. However, contamination by cell debris, although reduced following ultracentrifugation, was noted in the recovered particles.

The refined method developed in the present study recovered uncontaminated UHMWPE particles from tissues around failed total hip replacements for morphologic and quantitative SEM image analysis.

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MATERIALS AND METHODS

Periprosthetic tissues from nine failed total hip replacements were studied. These included three titanium alloy porous-coated surface replacements and two cementless and four cemented cobalt chrome alloy total hip replacements, revised from 2–20 years postoperatively for aseptic loosening. Tissues included capsules, interface membranes, and granulomas from osteolytic sites. Tissue from a patient with osteoarthritis undergoing primary arthroplasty was processed to serve as a control for the appearance of contaminating biologic or reagent particles under SEM.

The nine revised UHMWPE cups showed various degrees of gross wear damage, including polishing, scratching, and pitting. The femoral heads also showed damage, ranging from isolated scratches to gross dulling. Histologic sections of the sampled tissues indicated the presence of UHMWPE wear particles by polarized light examination (i.e., the appearance of birefringent needles from 1 ~ 10 μm) and positive Oil Red O staining[9–11] (i.e., pink, granular appearance of the cytoplasm of macrophages and giant cells, as well as pink-stained needles of the material that appeared birefringent under polarized light).

Tissue digestion

Approximately 5 g of periprosthetic tissues were diced and added to 10 cc of 5 N NaOh and digested at 65°C for 1–5 h in a shaking water bath. In our previous tissue digestion experiments,[9] we noted that reagents and deionized water often contained small particles that could contaminate the biomaterial particles. Therefore, all digest solutions and water were triple filtered through 0.2-μm nylon filters (Nalgene, cat. no. 163). Furthermore, all tubes and containers used in these experiments were triple-rinsed in filtered water before use. To prevent contamination of the low density particle fraction by lipids, the tissues were immersed in a 2:1 chloroform/methanol mixture for 24 h before digestion.

Isolation of PE particles

The digested material was centrifuged over a variable density gradient comprising 2.0 ml each of 5, 10, 20, and 50% sucrose, which had been filtered through a 0.2-μm filter. Three milliliters of the digest were placed on the top of the gradient, and the tubes were spun at 40,000 rpm, at 5°C for 3 h in a Sorvall OTD50B ultracentrifuge using an SW40Ti rotor.

The top layer of the gradient, containing only the lowest density particles, was collected by pipetting into cleaned 15-ml glass-capped vials. Particles were then washed free of sucrose by adding 30 cc of hot filtered water to the tubes, which were agitated for 1 h at 60°C. The vial contents were then transferred to clean ultracentrifuge tubes and topped with 2 cc each of 0.96 g/cm^3 and 0.90 g/cm^3 isopropanol. The tubes were ultracentrifuged for 1 h at 40,000 rpm at 25°C. The particles at the interface between the two isopropanol solutions were collected and stored in cleaned vials for characterization.

Particle examination

A 100—300 μl sample of the recovered particle solution was added to 2 cc of water, then filtered through a 0.2-μm Nucleopore polycarbonate filter (Costar, cat. no. 111106) using low vacuum pressure until nearly all of the water had been removed. The filter was then completely dried by evaporation under a light. The dried filter was attached to a standard SEM stub using double-sided tape, sputter-coated with gold palladium, and viewed with a Zeiss DSM 960 SEM. Images were transferred to a computer-based digital image analysis system (Image One, West Chester, PA). Individual rounded particles were "painted" manually before automated measurement of area and diameter. The length of individual elongated particles was traced with a cursor. Because it was not our intention to compare particle morphologies from the various hip replacement designs, the data were presented as the mean, median and standard deviations of the combined group.

Verification of particle identity

Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) were performed on approximately 5–10 mg of dried particles from each of five cases, as well as the pooled particles from four cases in which the individual amounts were insufficient to measure. A Mattson Polaris FTIR was used in the reflection mode and spectra were collected using 32 scan summations at a resolution of 16 cm^{-1}. A Perkin-Elmer DSC 4 instrument was used with an indium standard. The dried particles were melted at a heating rate of 10°C/min to 170°C, then cooled to 50°C.

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If a telephone interview would be of assistance in advancing prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone at the number provided below.

No fee is deemed necessary in connection with the filing of this Communication. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 50-1891.

Respectfully submitted,

RESULTS

Tissue digestion and particle examination

The more fibrous the tissue and the larger the pieces, the longer it took to digest. However, longer digestion times did not appear to damage the particles. The sucrose gradient employed in this study varied from approximately 1.23 g/cm^3 (50%) to 1.01 g/cm^3 (5%). This effectively separated the lighter PE particles (density approximately 0.94 g/cm^3) in a white or tan-colored band at the top of the sucrose, from the heavier cell debris and metallic wear particles, which formed a pellet at the bottom of the tube or remained in suspension. The second centrifugation step with an isopropanol gradient provided a smaller density gradient (0.90–1.0 g/cm^3), which further separated UHMWPE into a diffuse, white band between the two isopropanol solutions. Under polarized light, all of this material was birefringent, but, although individual needle-shaped particles several microns in length could be seen, the majority of the material was too small to be clearly resolved (Fig. 1).

Scanning electron microscopy of these particles revealed well-dispersed individual or small groups of particles that showed two consistent morphologies, rounded or elongated (Fig. 2). Image analysis of the rounded particles showed that they ranged from 0.07–6.3 μm in diameter (average 0.38 ± SD 0.32 μm, median 0.3 μm, number imaged = 882), and the elongated shaped particles ranged from 0.57–12.2 μm in length (2.19 ± 1.26 μm, median 1.9 μm, N = 362). The majority (96.6%) of round particles were submicron in size. The remainder were approximately 1 μm in diameter, although occasionally larger particles, (possibly groups of smaller sized particles) were seen.

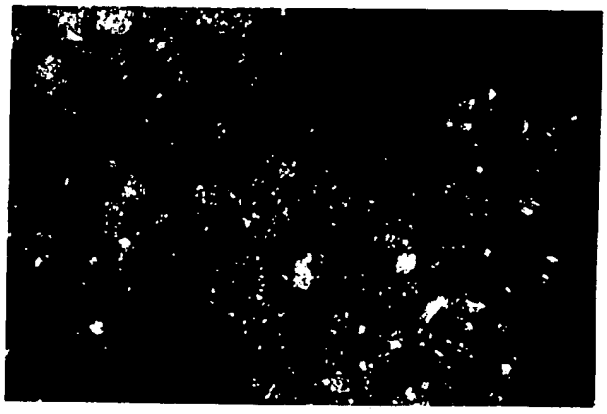

Figure 1. Polarized light micrograph (×60) of material recovered from between the two isopropanol layers following the second centrifugation step. All of this material is birefringent but most of the particles are too small to be seen clearly.

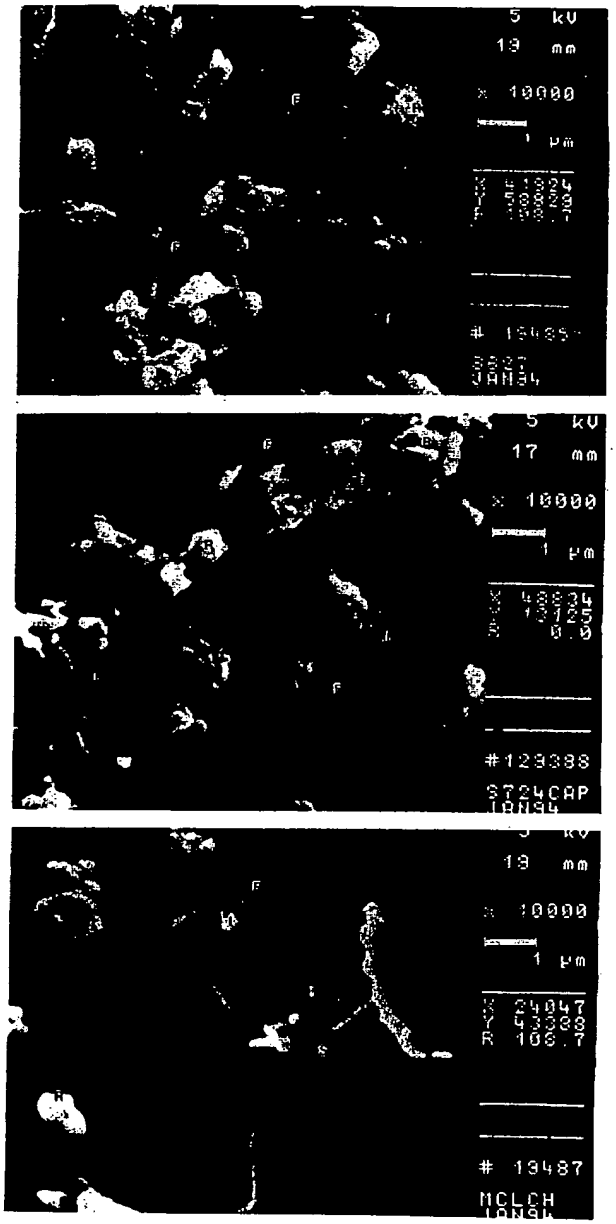

Figure 2. Scanning electron micrograph of particles recovered from the isopropanol gradient. The background is a 0.2-μm filter. Particles were isolated from tissues surrounding: (a) Cemented Charnley THR, (b) pressfit ATH THR, (c) porous-coated titanium alloy surface replacement. Each shows thin, tapered fibrils (F), irregularly rounded particles approximately 1 μm in diameter (R) and individual submicron-size particles (I) either lying on the filter or on the surface of the larger particles. A larger shred (S) is seen in (c). (×10,000).

There were two forms of elongated particles: "fibrils" were relatively short and thin (0.5–3 μm long, < 0.25 μm thick). In contrast, "shreds" were up to 12 μm long and up to 1 μm thick. Ninety percent of the elongated particles were >1 μm in length, but

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<5% were >5 μm in length. The fibrils were often seen with rounded "heads" at one end and a thin taper at the other. The shreds were often tapered, but only occasionally had heads. Occasionally, irregularly shaped flakes of material were noted, but these comprised <1% of the imaged particles. Cell debris and other contaminants were only rarely seen among the recovered polyethylene particles.

The FTIR spectrum of the recovered material was consistent with UHMWPE—that is, with peaks around 2917, 2850, 1470, and 721 cm^{-1} [12] (Fig. 3). Similarly, DSC of the recovered particles showed a melting temperature of 133°C, also typical of polyethylene.

DISCUSSION

It was not the intention of our study to correlate the particle morphology with the different types of hip replacement. Rather, our aim was to assess the reproducibility of a method for UHMWPE particle isolation in a number of cases where the nature of the tissues and the particle populations differed (with or without cement, differing metal alloys). In all nine cases, particle morphology was very consistent, being either rounded or elongated in shape with two forms of each being noted. In a separate study, we compared the size and appearance of recovered particles with features seen on the surface of the associated polyethylene cups, and formulated the hypothesis that the fibrils may have been the result of microadhesion or abrasion, whereas the larger shreds were the result of third-body wear.[13]

Shanbhag et al. digested periprosthetic tissues with potassium hydroxide and performed an extensive separation procedure[14] to obtain polyethylene particles reported to be spheroid in shape and 0.1–2.0 μm in mean dimension. However, their technique resulted in carpet-like aggregates of particles that precluded the identification of individual particles in three of the 10 cases studied, and made morphologic analysis difficult in the remaining cases. We applied Shanbhag's technique to additional samples of tissues previously characterized by the method presented in the current study and also noted severe clumping of the particles such that individual morphologies could not be clearly discerned. However, individual particles (both rounded and fibrillar) were found in the discarded supernatant solutions. The loss of particles in this manner is probably due to the use of low centrifuging forces (1000 × *g*, approximately 3000 rpm on our centrifuge). The technique described in the present study employs ultracentrifugation (40,000 rpm) because high centrifugal forces are required to remove all of the submicron-size polyethylene particles from suspension.[15]

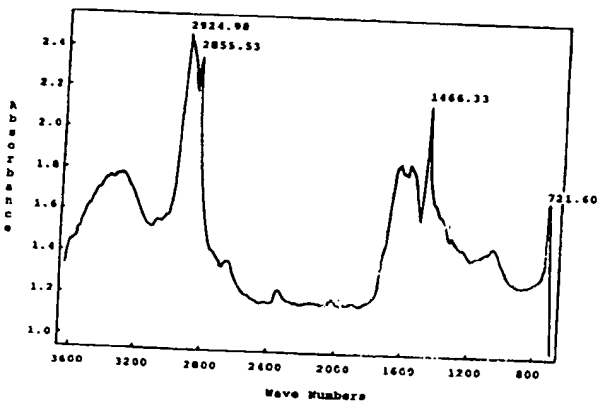

Figure 3. Fourier transform infrared spectra of the particles recovered from the isopropanol showing peaks consistent with UHMWPE.

The quantitation of recovered polyethylene particles remains problematic because of their small size and dual morphology. An automated particle counter (Coulter Multisizer, Hialeah, FL) was applied to counting and sizing particles isolated following tissue digestion.[16] However, this instrument is limited to particles that are larger than approximately 0.5 μm, and assumes that particles are spherical. Only a small proportion of the particles recovered in the present study met those criteria.

A submicron particle analyzer, the Coulter N4MD, was evaluated previously for particle quantitation.[17] This instrument uses photon correlation spectroscopy to size particles ranging from 3 nm to 3 μm. However, accurate sizing requires that the particles must be separated in size by at least a factor of 3. SEM of recovered UHMWPE particles suggests that their size range is too broad to be sized accurately by photon correlation spectroscopy. A further limitation of both instruments is their inability to discriminate between biomaterial particles and contaminants, as well as individual particles or clumped particles. Characterization of particle size by SEM is a more reliable method because morphologic criteria for the rejection of contaminant or clumped particles can be applied.

The isolation protocol described in this study required specialized ultracentrifugation equipment but was otherwise relatively simple. It involved fewer steps and less time than the method of Shanbhag et al.,[14] while avoiding the problems of clumping noted with that method, as well as with our previous protocol.[9] Digested tissue that had not been processed by gradient separation[18] was found to be unsuitable for analysis by SEM as the particles were almost totally masked by the dried contaminants. It follows that this material would also be unsuitable for cell culture studies.[19] Only well-characterized particles of

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high purity should be used for this purpose. The modified protocol described in the present study is suitable for the collection of such particles.

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